

EXHIBIT 4

Mitochondrial C-tract Alteration in Premalignant Lesions of the Head and Neck: A Marker for Progression and Clonal Proliferation

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ABSTRACT

Purpose: Although mitochondrial DNA mutations have been described recently in many different tumor types, the nature and timing of such alterations remain unclear. In an effort to further examine the role of mitochondrial DNA mutations in carcinogenesis, we examined 137 premalignant lesions of the head and neck from 93 patients for DNA alterations in the poly-cytosine tract (C-tract) of the displacement loop, discovered recently to be a hot spot of mitochondrial DNA alteration.

Experimental Design: All premalignant lesions were tested using a length-based PCR assay, which amplified the C-tract region of mitochondrial DNA. Somatic microsatellites at six loci were also tested on a subset of patients with metachronous or synchronous lesions found to possess a mitochondrial C-tract alteration.

Results: Thirty-four of 93 (37%) patients harbored lesions that displayed a C-tract alteration. There was a clear increase in incidence from histologically benign hyperplasia (22%) to squamous carcinoma *in situ* (62%; $P < 0.01$). We also tested synchronous dysplastic lesions, metachronous dysplastic lesions, and normal epithelium adjacent to dysplastic epithelium with this assay. In most cases, the mitochondrial C-tract status identified a clonal relationship between these lesions. Genomic microsatellites also confirmed that a clonal relationship was present in many of these cases.

Conclusions: Mitochondrial DNA alterations in the head and neck occur in the earliest premalignant lesions and demonstrate a rising incidence that parallels histological severity. These alterations are valuable as additional markers of histopathological progression.

INTRODUCTION

Mitochondrial DNA alterations have been studied in many different tumor types, including stomach (1–3), colon (4–6), pancreas (7), kidney (8), breast (9), bladder, lung, and head and neck cancers (10). Some of these tumors (20–60%) display alterations within the mitochondrial genome. These mutations have also been discovered in paired bodily fluids of patients with tumors having known mitochondrial alterations (11). Although alterations may occur throughout the mitochondrial genome, the C-tract² (between nucleotides 303 and 315) located in the D loop of the mitochondrial genome has emerged as a locus of increased polymorphism in the general population and mutation in primary tumors. The D loop functions as a promoter for both the heavy and light strands of the mitochondrial DNA but does not encode any functional proteins. In normal tissues from the general population, there are between seven and nine cytosine residues in this C-tract (12). A recent study indicated that several different solid tumor types have a propensity toward expansions/deletions in this particular location, including 41% of head and neck squamous cell carcinomas (10).

The significance of a mutation in this region has not yet been elucidated. Because normal tissue contains such polymorphic expansions/deletions (13, 14), it is hypothesized that these mutations may simply be markers of clonal growth, rather than true functional alterations. Indeed, several studies have suggested that the baseline frequency of mitochondrial genome alterations is higher compared with the nuclear genome because of a lack of histone packaging (15, 16), decreased polymerase fidelity (17), and increased exposure to toxic free-oxygen radicals in the mitochondria (18). Coller *et al.* (19) put forth a model that suggests that tumors can display homoplasmic mitochondrial mutations as a result of random segregation. Although the mechanism is not well understood, the frequent transformation of mitochondria to homoplasmicity in a tumor cell provides a potential marker to detect clonal outgrowth. The detection of homoplasmic mitochondrial alterations could potentially be used to track tumor development and spread.

To further examine the frequency and timing of mitochondrial C-tract alterations, we tested premalignant lesions of the head and neck. Premalignant lesions in the head and neck have emerged as an excellent model for tumorigenesis given their easy accessibility and documented histological progression, accompanied by the accumulation of molecular alterations within the somatic genome. Several investigators have noted a predictable pathway of chromosomal loss that correlates with histopathological progression (20). These alterations occur even in

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² The abbreviations used are: C-tract, poly-cytosine tract; D loop, displacement loop; OR, odds ratio; CI, confidence interval.

the earliest of lesions and appear to be related to tumor progression (21, 22).

Thus, we examined oral cavity leukoplakias for mitochondrial C-tract alterations. Histopathologically, these lesions ranged from simple hyperplasias without dysplasia, to mild dysplasia, moderate dysplasia, severe dysplasia, and carcinoma *in situ*. To determine whether the alteration was conserved for metachronous and synchronous lesions, we further examined a subset of lesions that demonstrated histopathological progression over time, as well as lesions that were synchronous but noncontiguous. To further examine the clonal relationship of mitochondrial alterations, normal epithelium adjacent to dysplasias was also examined, and genomic microsatellites were performed on the synchronous and metachronous lesions found to have mitochondrial alterations.

MATERIALS AND METHODS

Tissue. The surgical pathology files of the Johns Hopkins Hospital were searched for consecutive cases of dysplasias of the upper aerodigestive tract without a previous or concurrent head and neck malignancy over a 28-year period. Samples (137) from 93 patients were identified (18 patients had synchronous lesions, and 7 patients had metachronous lesions). From each case, at least one representative tissue block was selected for lesional microdissection and DNA extraction.

Histopathologic Assessment of Dysplasia. For each case identified through the search of the surgical pathology files, the H&E-stained slides were reviewed by a head and neck pathologist with extensive experience in grading dysplasias of the upper aerodigestive tract (W. H. W.). The degree of dysplasia was graded as according to the guidelines established by the WHO (23).

DNA Extraction. Fifteen 10- μ m sections were cut from paraffin blocks and microdissected using the H&E slide as a guide. Uninvolved submucosa was microdissected as a representative normal. The samples were then placed in xylene for 12 h and centrifuged at 13,500 rpm. The tissue pellet was collected and digested in sodium dodecyl sulfate/proteinase K over the next 48 h at 48°C. The product was then phenol-chloroform extracted and stored at -20°C. The samples were diluted 50-fold and then analyzed by PCR.

Mitochondrial Microsatellite Analysis. Normal and premalignant lesion pairs were run using specific primer pairs to amplify a 109-bp region, including the C-tract within the D loop of the mitochondrial genome. The forward primer sequence was 5'-ACAATTGAATGTCTGCACAGCCACTT-3', and the reverse sequence was 5'-GGCAGAGATGTGTTTAAGTGCTG-3'. The forward primer was then labeled with γ -ATP. After a denaturation step at 95°C for 2 min, samples were subject to 35 cycles of the following conditions: 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The amplified product was then run on a 6% denaturing polyacrylamide gel. The gel was exposed to radiographic film and analyzed.

The analysis of the normal/tumor samples consisted of single bp deletions/expansions, as well as those exceeding 1 bp. With alterations exceeding 3 bp, alterations were determined by comparison with controls to determine the amount of loss or

Table 1 C-tract mutations by site

Stage	Freq.	Site	Alteration (bp)
Hyperplasia without atypia	7/32	R tongue	-1
		Buccal mucosa	+1
		R tongue	+1
		Tongue	+1
		L tongue	+1
		L buccal mucosa	+1
		FOM	+1
Mild dysplasia	3/9	L vocal cord	+1
		L tongue	+2/+3
		p.o. cavity	-10
Moderate dysplasia	9/25	Soft palate	+1
		Larynx	+1
		Buccal mucosa	+1
		FOM	+1
		L tongue	+1
		R buccal mucosa	+1
		R tonsil	+1
		Tongue	+2
		Dorsal tongue	+2
		Dorsal tongue	+1
Severe dysplasia	7/14	L subglottis	+1
		L vocal cord	+1/+2
		R vocal cord	+1/+2
		L vocal cord	-3
		Soft palate	-4
		R tongue	-5
		Retromolar trigone	-1
		L tongue	+1
Carcinoma in situ	8/13	Ventral tongue	+1
		FOM	+1
		L FOM	+1
		R buccal mucosa	+1
		FOM	+3
		Hard palate	+4

gain. Those samples deemed to be positive for an alteration were reconfirmed independently by two observers [P. K. H. and J. A. C.] by separate PCR amplification. Of note, the samples displaying mitochondrial alterations may also be accompanied by the normal band, indicating either contamination with normal DNA, or a variation in a heteroplasmic state.

Microsatellite Alterations. The 7 patients with metachronous and synchronous samples found to contain mitochondrial alterations were tested for concomitant genomic microsatellite alterations at six loci (D3S1286, D3S1289, IFNA, D9S162, D9S161, and TP53). The PCR conditions used were described previously (24), and the products were run on a 6% denaturing polyacrylamide gel.

Statistical Methods. The major statistical end point in this study was the association of mitochondrial DNA mutations with histopathological diagnosis of premalignant head and neck lesions. Patients were categorized into five groups according to lesion progression: (a) hyperplasia without dysplasia; (b) mild dysplasia; (c) moderate dysplasia; (d) severe dysplasia; and (e) carcinoma *in situ*. The association of mitochondrial alteration with increasing severity of dysplasia was based on a cross-tabulation and a logistic regression model (25). All statistical computations were performed using the SAS system (26), and all *P*s reported are two sided.

Table 2 Summary of mitochondrial alterations by histologic grade

Grade	Overall frequency	Alterations ± 1 bp	Alterations $> \pm 1$ bp	OR ^a (95% CI)
Hyperplasia without dysplasia	7/32 (21.9%)	7/7 (100%)	0/7 (0%)	1.0
Mild dysplasia	3/9 (33%)	1/3 (33%)	2/3 (67%)	1.8 (0.35, 9.02)
Moderate dysplasia	9/25 (36%)	7/9 (78%)	2/9 (22%)	2.0 (0.62, 6.47)
Severe dysplasia	7/14 (50%)	2/7 (29%)	5/7 (71%)	3.6 (0.93, 13.66)
Carcinoma <i>in situ</i>	8/13 (61.5%)	6/8 (75%)	2/8 (25%)	5.7 ^b (1.4, 23.0)
Total:	34/93 (37%)	23/34 (68%)	11/34 (32%)	

^a ORs determined by comparing each histologic category with hyperplasia without dysplasia.

^b Statistically significant: $P < 0.01$.

RESULTS

Mutations by Histological Grade. DNA from 137 isolated premalignant lesions in 93 patients with absence of prior or concurrent malignancy was analyzed for mitochondrial C-tract alterations. Some of these lesions (74) were from the oral cavity, 5 from the oropharynx, and 14 were from the larynx (Table 1). After histopathological review, there were 32 hyperplasias without dysplasia, 9 mild dysplasias, 25 moderate dysplasias, 14 severe dysplasias, and 13 carcinoma *in situ* lesions. For patients with multiple biopsy samples, the most histologically severe sample was assayed so as not to overestimate the alteration rate for low-grade lesions. Overall, 34 of 93 (37%) patients harbored premalignant lesions that exhibited some form of somatic C-tract alteration when compared with uninvolved germ-line DNA. Fig. 1 demonstrates sample gels with altered C-tract regions.

The probability of mitochondrial alterations increased with increasing severity of dysplasia (Table 2). Compared with the hyperplasia without dysplasia group, those with mild and moderate dysplasia were approximately two times as likely to have mitochondrial alterations, ORs 1.8 (95% CI: 0.35, 9.02) and 2 (95% CI: 0.62, 6.47), respectively. The risk in the severe dysplasia group was estimated to be ~ 3.6 (95% CI: 0.93, 13.66) times the risk in the hyperplasia without dysplasia group. Carcinoma *in situ* had the highest probability of alterations compared with hyperplasia without dysplasia, OR 5.7 (95% CI: 1.4, 23), $P = 0.01$. Of note, when the severe dysplasia and carcinoma *in situ* groups were combined, the OR was statistically significant as well [4.46 (95% CI: 1.4, 23), $P = 0.01$].

Of the 34 cases with C-tract alterations, 23 of 34 (68%) had single bp alterations, and 11 of 34 (32%) of cases had multiple bp alterations. Within the different histological categories, the likelihood of a single bp *versus* multiple bp alteration did not correlate with histological grade.

Metachronous Lesions. Seven patients had multiple biopsy samples of recurrent lesions, and all 35 of these samples were analyzed for the C-tract alteration (Table 3). Three of these patients had lesions with a mitochondrial C-tract alteration, and all three of these molecular alterations persisted. However, patient 3 did not have the alteration on his third and final biopsy, which was performed 2 years after his initial procedure.

Synchronous Lesions. Eighteen patients underwent biopsies of synchronous but clinically noncontiguous leukoplakias of the mucosa lining the upper aerodigestive tract. Ten of 18 patients harbored a lesion that demonstrated an alteration in the C-tract. Of these 10 patients, 8 exhibited biopsies that, regard-

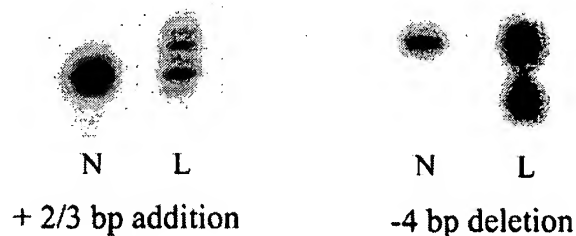


Fig. 1 Representative gel photographs of mitochondrial C-tract alterations demonstrating detection of mitochondrial C-tract alteration by a length-based PCR assay run on a 6% polyacrylamide gel. Insertion/deletion lengths are determined by use of shadow bands. N = normal control, L = premalignant lesion.

less of site, showed the identical pattern of mitochondrial alteration.

Adjacent Epithelium. Normal epithelial tissue adjacent to dysplastic epithelium was microdissected in eight cases. Three of the eight cases of dysplasia demonstrated a mitochondrial C-tract alteration, and in all of these cases, the histologically "normal" adjacent epithelium demonstrated the identical pattern of expansion/deletion as the premalignant lesion (data not shown).

Genomic Microsatellite Data. In an effort to determine the clonal relationship between the synchronous and metachronous lesions, seven samples found to contain mitochondrial alterations were tested for alterations at six different microsatellite markers. These markers were selected because of their known alteration in premalignant lesions. Three of seven samples displayed features of clonality, two of seven were noninformative at these loci, and two of seven were indeterminate, showing characteristics of loss in one lesion at one microsatellite locus but not in the others. Fig. 2 demonstrates a representative sample of a patient with two metachronous lesions and the mitochondrial alterations and microsatellite alterations (CFS1-R) displayed side by side. Fig. 3 shows the corresponding histology to these same lesions.

DISCUSSION

Premalignant mucosal lesions in the head and neck provide an excellent model of cancer progression because of the well-described molecular progression pathways and defined histopathological appearance. Within the current histological staging system, these lesions are broken down into hyperplasia

Table 3 Table depicting longitudinal tumor progression of normal/tumor pairs and summary of mitochondrial C-tract alteration

These patients received at least three and up to five biopsies on different dates. The entries show the date of procedure, histologic grade, biopsy site, and nature of mitochondrial DNA alteration. Patients 1, 6, and 7 show a preservation of the alteration despite having biopsies taken at different timepoints.

Patient	Biopsy 1	Biopsy 2	Biopsy 3	Biopsy 4	Biopsy 5
1	5/95 Moderate dysplasia Larynx +2 bp 10/99	12/96 Severe dysplasia L vocal cord +2 bp 10/99	12/96 Severe dysplasia R vocal cord +2 bp 7/00		
2	Mild dysplasia L FOM 0 bp 4/00	Moderate dysplasia FOM 0 bp 6/00	Moderate dysplasia Lateral tongue 0 bp 6/00		
3	Moderate dysplasia L ant/vent tongue 0 bp 3/98	Severe dysplasia L tongue 0 bp 4/99	Moderate dysplasia L tongue 0 bp 2/00		
4	Moderate dysplasia R post tongue 0 bp 2/00	Moderate dysplasia R post tongue 0 bp 3/00	Moderate dysplasia R lat tongue 0 bp 9/00	Mild dysplasia FOM 0 bp 10/00	Moderate dysplasia R lat tongue 0 bp
5	Moderate dysplasia R tongue 0 bp 2/97	Moderate dysplasia R tongue 0 bp 6/98	Mild dysplasia R tongue 0 bp 12/99	Moderate dysplasia Ventral tongue 0 bp 4/00	
6	Severe dysplasia Tongue +1/+2 bp 6/92	Severe dysplasia R lat tongue +1 bp 7/92	Moderate dysplasia Dorsum tongue +1 bp 5/94	Severe dysplasia Tongue +1 bp	
7	Hyperplasia without dysplasia L tongue +1 bp	Hyperplasia without dysplasia L tongue +1 bp	Hyperplasia without dysplasia L tongue 0 bp		

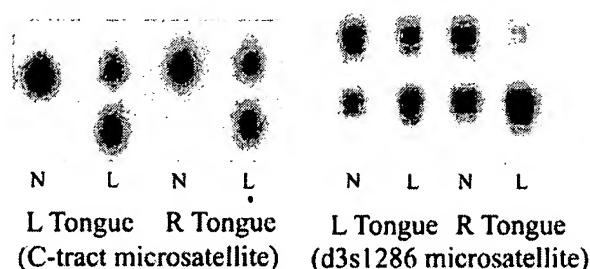


Fig. 2 Mitochondrial C-tract microsatellite and genomic microsatellite (*d3s1286*) gels from samples obtained from the same patient with synchronous areas of leukoplakia (mild dysplasia and severe dysplasia) on anatomically separate regions of the tongue, demonstrating alterations in both the mitochondrial and somatic genome. *n* = normal control, *L* = premalignant lesion.

without dysplasia, dysplasia (mild, moderate, and severe), and carcinoma *in situ*. These lesions are biopsied frequently because of their propensity to progress to malignancy, although the hyperplastic lesions without dysplasia have only a small chance of malignant transformation (27). Genomic microsatellite markers have been studied extensively in these lesions, generating a widely accepted genetic progression model for head and neck squamous cell carcinoma (20). In the context of this model, specific chromosomal losses predict the likelihood of malignant

transformation (21, 22). These and other studies suggest that there is an early onset of genetic alteration, and the accumulation of genetic errors is associated with increasing malignancy.

The data presented here suggest that, similar to genomic DNA, mitochondrial DNA is also subject to genetic alterations early in the progression of head and neck lesions. Overall, 37% of premalignant lesions harbored a C-tract alteration. The prevalence increased from benign hyperplasia to dysplasia and carcinoma *in situ*, implying that the mitochondrial C-tract mutation may be a useful marker for malignant progression, although additional studies with clinical correlation need to be performed.

The relationship between multiple head and neck cancers from the same patient has been studied by Bedi *et al.* (28). By using X-chromosome inactivation and microsatellite analysis, it was shown that the multiple tumors arose from a single clone. A clonal relationship between metachronous and synchronous premalignant lesions was also identified in our study using mitochondrial alterations as a genetic marker in these patients. The identical pattern of C-tract alteration was conserved in 8 of 10 patients with synchronous lesions and in 2 of 3 patients with metachronous lesions. Normal epithelium adjacent to dysplastic epithelium also exhibited the identical pattern of C-tract alteration. Furthermore, genomic microsatellites at six loci were performed in 7 patients with synchronous and metachronous lesions demonstrating mitochondrial alteration, of which 3 of 7 clearly displayed common alterations in at least one of the markers, suggesting that mitochondrial C-tract alterations are markers of clonality. The few samples that did not exhibit the



Fig. 3 H&E photomicrographs corresponding to the synchronous left and right tongue lesions in Fig. 2, demonstrating cellular characteristics consistent with: (a) mild dysplasia from left tongue; and (b) severe dysplasia from right tongue.

same pattern may represent an independent lesion of a different clonal origin, or the same clone that diverged earlier on the progression pathway, before mitochondrial alteration.

The mechanism underlying homoplasmic mitochondrial DNA alterations and the potential role of these alterations in tumorigenesis is still poorly understood. One possibility for the observed increase in C-tract alterations with advanced histological grade is that there is some selective growth advantage conferred to both the mitochondria and the cell. In this scenario, the mitochondrial DNA will eventually become a homoplasmic population because of a selection advantage. Polyak *et al.* (5) found, in colon cancer cell lines, a propensity toward homoplasmy within the mitochondrial genome. Using cell fusion models, mutated mitochondria quickly dominated and became homoplasmic, perhaps because of selective pressures. However, a selective growth advantage because of an alteration is less likely in this hot spot region, because the C-tract is known to be polymorphic and contain between seven and nine cytosine residues within the normal population (29). The majority of the mutations found in our study involved a small 1 bp expansion or deletion. Because this alteration of 1 or 2 bp is still part of the wild-type distribution, the functional difference as a result of this mutation remains unclear.

It is also possible that C-tract alterations are markers for

defects in the cellular maintenance of DNA integrity that provides a selective replication advantage for the cell, within which the mitochondrion resides. Mitochondrial C-tract alterations would then be seen as epiphenomena related to alterations in the maintenance of genetic stability. Therefore, C-tract alterations would occur without necessarily indicating a change in the function of the mitochondrial C-tract. In hereditary colon cancer, defects of mismatch repair proteins lead to diffuse microsatellite instability. In at least one case, a prostate tumor with nuclear microsatellite instability was found to harbor multiple mitochondrial mutations (30). To account for common mitochondrial repeat/alterations, a defect in mitochondrial DNA mismatch repair mechanisms has been theorized (4), although no human mitochondrial *MMR* gene has been discovered. Therefore, it is also plausible that maintenance genes in the nuclear genome may also play a role in the maintenance of mitochondrial DNA integrity.

Finally, there is also the possibility that mitochondrial homoplasmy occurs simply by chance. It has been demonstrated using *in vitro* and computer models that mitochondria may progress to a homoplasmic population simply by clonal selection of a particular cell (19). The data here show that there is an increased likelihood of homoplasmic mitochondrial alteration with histological progression toward overt malignancy. Increasing waves of clonal expansion associated with tumor progression could explain this observation.

Our data show that the mitochondrial C-tract alteration is an early event, with an increased likelihood of alteration as the histological grade increases in head and neck premalignant lesions. Furthermore, these alterations may be used as markers of clonal progression from premalignancy to cancer or to establish a clonal relationship between multiple lesions. Additional studies need to be conducted to determine the mechanistic role of these alterations in cancer progression and the potential clinical significance of this assay.

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